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Quantitative Analysis of Oral Movements in a Mouthbrooding Chichlid, Orcochromis esculentus

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QUANTITATIVE ANALYSIS OF ORAL MOVEMENTS IN A MOUTHBROODING CICHLID, *OREOCHROMIS ESCULENTUS*

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This thesis is submitted in partial fulfillment of

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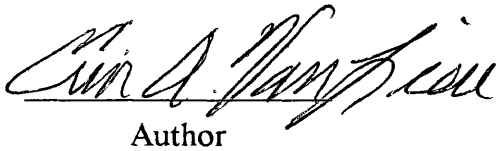

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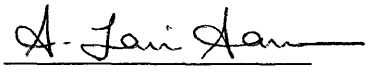
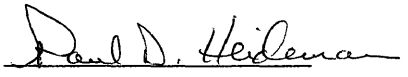
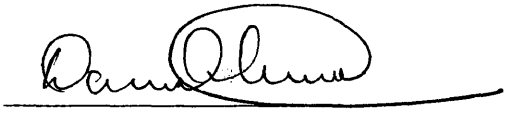


S. Laurie Sanderson



Paul Heideman



Daniel Cristol

Dedication

This thesis is dedicated to my parents Edna and Edward Van Lieu who have supported me every step of the way and have always believed in my abilities.

TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Endoscopic analysis was used to describe the behavior of the young in the oral cavity of mouthbrooding *Oreochromis esculentus* in response to oral movements of the parent, and to assign days of the mouthbrooding period to four developmental periods in the young. These developmental periods were then used for quantitative analysis of parental churning and pumping variables. This analysis showed that the mean time per churn bout, mean number of churns per bout, mean time spent churning, and the mean number of churns per 100 sec tended to peak at hatching. The mean time per churn, mean time per pump and the mean number of pumps per 100 sec tended to reach minimum values at hatching. The mean time per pump bout and the mean number of pumps per bout tended to decrease throughout the mouthbrooding period, while the mean number of churn bouts per 100 sec tended to increase toward the end of the mouthbrooding period. These patterns were similar to those reported for oral movement variables in the two mouthbrooding teleost species studied previously. Patterns of churning in *O. esculentus* were also compared to patterns of fanning reported previously for substrate brooding species. Similar to churning variables, fanning variables tended to peak at hatching. These results support the hypothesis that churning in *O. esculentus* serves the same function as churning in other mouthbrooding species, and that churning serves the same function as fanning in substrate brooding species. These findings are consistent with the hypothesis that churning and fanning serve to provide oxygen to the young and to remove waste from the oral cavity.

QUANTITATIVE ANALYSIS OF ORAL MOVEMENTS IN A MOUTHBROODING CICHLID, *OREOCHROMIS ESCULENTUS*

Introduction

In all known members of the family Cichlidae, composed of over 1,000 species (Keenleyside, 1991), one or both parents care for the fry from spawning until independence (Balshine-Earn and Earn, 1998; Keenleyside, 1991; Kuwamura and Mihigo, 1988). A prolonged association between parents and offspring is unusual among teleost families (Balshine-Earn and Earn, 1998; Keenleyside, 1991; Lavery and Reebs, 1994), only 21% of which practice some form of parental care (Balshine-Earn and Earn, 1998; Goodwin et al., 1998; Gross and Sargent, 1985).

Cichlids can be divided into two reproductive classes based on the form of post-fertilization care: (1) substrate brooding and (2) mouthbrooding (Keenleyside, 1991; Mrowka, 1983; Yanagisawa, 1986). Substrate brooders are typically monogamous, biparental species which lay adhesive eggs on a firm substrate and guard the young (Mrowka, 1983; Keenleyside, 1991). Mouthbrooders are normally polygamous species in which the female lays non-adhesive eggs and incubates the young in the oral cavity (Keenleyside, 1991; Mrowka, 1983).

Mouthbrooding has been described in ten families of teleost fishes (Yanagisawa and Sato, 1990) and is thought to have evolved from biparental substrate brooding (Balshine-Earn and Earn, 1998; Goodwin et al., 1998; Pouyaud and Agnèse, 1995) under conditions where suitable substrate for nesting is scarce, competition for nesting sites is intense, or predation levels are high (Baylis, 1981; Keenleyside, 1991; Mrowka, 1983; Yanagisawa

and Sato, 1990). In addition, gas exchange of the young may have been enhanced compared to substrate brooding (Keenleyside, 1991; Mrowka, 1983). Behaviors associated with mouthbrooding can be seen in many substrate brooders that use their oral cavities to transport the young between nest sites and to return stray individuals to the defended nest (Balshine-Earn and Earn, 1998; Gittleman, 1981; Keenleyside, 1979; Mrowka, 1983).

Rapid oral movements are commonly seen in fish when they have objects, such as food, gravel, or eggs, in the oral cavity (Oppenheimer and Barlow, 1968; Oppenheimer, 1970). This behavior, hereafter churning, has been referred to as swallowing, mumbling, gargling, rolling, chewing, and churning (Keenleyside, 1979) and has been reported for both mouthbrooders (Oppenheimer and Barlow, 1968; Pinheiro, 1980; Timms and Keenleyside, 1975) and substrate brooders (Baerends and Baerends-Van Roon, 1950; Oppenheimer, 1970; Walker, 1964) when eggs are in the oral cavity (Oppenheimer, 1970).

Before the young are free-swimming inside the oral cavity of the parent they may form a multi-layer heap. Churning probably moves the young within the oral cavity such that those at the bottom are brought to the top where they are washed free of wastes and exposed to a current of fresh water (Oppenheimer and Barlow, 1968; Oppenheimer, 1970; Timms and Keenleyside, 1975). The other major type of oral movement exhibited by mouthbrooders during the brooding period is pumping, a normal ventilatory movement. Pumps and churns alternate in bouts of varying duration during the brooding period.

Quantitative analysis of churning bout characteristics has revealed a peak in most

variables at hatching, suggesting that the primary function of churning is oxygenation of the young (Keenleyside, 1991; Oppenheimer and Barlow 1968; Oppenheimer 1970; Timms and Keenleyside, 1975). An increase in churning variables from spawning to hatching may be the result of the increasing oxygen demands of the young (Oppenheimer and Barlow, 1968; Oppenheimer, 1970). The decline in churning variables after hatching may be because the young start their own ventilation and are able to lift themselves above the boundary layer where they can receive oxygenated water (Keenleyside, 1991; Oppenheimer and Barlow, 1968; Oppenheimer, 1970; Timms and Keenleyside, 1975).

Substrate brooders exhibit an activity called fanning, where the parent maintains a position close to the nest and fans the brood with large amplitude movements of the pectoral fins (Keenleyside, 1991; Bakker and Mundwiler, 1999; Takegaki and Nakazano, 1999). Fanning bout variables also tend to peak at hatching. Therefore, fanning is thought to be functionally similar to and the evolutionary precursor of churning (Keenleyside, 1991; Oppenheimer, 1970). Indirect evidence that fanning serves the same function as churning can be seen in delayed mouthbrooders that begin oral incubation at hatching. These species are considered to be intermediate between immediate mouthbrooders, who take the young into the oral cavity soon after spawning, and substrate brooders (Goodwin et al., 1998; Keenleyside, 1979; Keenleyside, 1991). Delayed mouthbrooders fan their eggs until hatching at which time churning is substituted for fanning (Dupuis and Keenleyside, 1982; Timms and Keenleyside, 1975).

This changing pattern of oral movements during the mouthbrooding period has been examined in only two mouthbrooding species: *Sarotherodon melanotheron* (Cichlidae), an immediate paternal mouthbrooder (Oppenheimer and Barlow, 1968) and *Aequidens*

paraguayensis (Cichlidae), a delayed biparental mouthbrooder (Timms and Keenleyside, 1975). However, fanning variables have been quantified for each day of the brooding period in eight substrate brooding species.

The subject of this study, *O. esculentus*, is an immediate maternal mouthbrooder (Onyari, 1983). I hypothesized that churning in *O. esculentus* serves the same function as churning in other mouthbrooding species and fanning in substrate brooding species. I predicted that churning patterns throughout the mouthbrooding period in this species would resemble churning patterns in *S. melanotheron*, and that these churning patterns would also resemble fanning patterns in substrate brooders.

This study may be a valuable contribution to this field because it addresses three questions regarding churning behavior and analysis of this behavior. (1) It is not known how the behavior of the young and the oral movements of the parent influence one another, and this question cannot be addressed until behavior of the young within the oral cavity is documented. This study is the first to investigate a direct relationship between parental behavior and young behavior during the mouthbrooding period. (2) The presumed function of churning is based on the patterns exhibited within one species. Churning should be investigated in other species to see if similarities allow presuming a common function. (3) In past studies, complex oral movements were viewed in real time. Frame-by-frame analysis in this study allowed oral movements to be viewed as many times as necessary to accurately describe the patterns of these movements

MATERIALS AND METHODS

Four female and one male specimen (approximately 22-27 cm standard length) bred at Boston University from pure stock native to Lake Kanyaboli in East Africa were held in each of two 280 liter aquaria. The temperature was maintained at 26-28° C. The fish were fed TetraMin flake food daily. The aquaria had gravel substrates (3-9 mm diameter) which allowed the males to create a depression in which the female could deposit her eggs. PVC pipes (12.0 cm i.d.) and a flower pot (20.0 cm i.d.) were placed at opposite ends of the aquaria to give the females refuge from the aggressive territorial behavior of the male.

The females were checked daily for mouthbrooding behavior. Mouthbrooders were identified initially by either the presence of churns in the absence of feeding, extension of the geniohyoideus muscle, and/or absence of feeding behavior when offered Tetramin flakes. Mouthbrooding was then confirmed visually by presence of eggs in the oral cavity. The first day that a mouthbrooder was detected was designated Day 1 of the brood. Each mouthbrooder was transferred into a 110 liter aquarium on Day 1 for the external analysis and Day 3 or 6 for the endoscopic analysis.

The temperature in these aquaria was maintained at 26-28° C. A porous plastic aquarium divider limited movement of the fish to one half of the aquarium. This helped to keep the fish within the field of view during videotaping, and to limit movement of the fish when the endoscope was in the oral cavity. To control the level of ammonia in the

aquaria, Nitra-Zorb pellets (7.4 oz) were placed in a bubble-up filter at the opposite end of the aquarium. *O. esculentus* is a filter-feeder (Onyari, 1983), therefore, it is possible that particles are ingested throughout the mouthbrooding period. In order to simulate the food particles present in a natural environment, flake food (0.2-0.8 g) was introduced every two to three days during the mouthbrooding period.

Endoscopic recordings and analysis

Activity inside the oral cavity was recorded throughout the mouthbrooding period to (1) provide insight into the function of these movements by describing behavior of the young within the oral cavity in response to oral movements and (2) to identify the following developmental periods: egg (developmental period 1), post-hatching (developmental period 2), pre-swimming (developmental period 3), and free-swimming (developmental period 4). These developmental periods were chosen because if oxygenation of the young is the primary function of churning, then there should be a marked difference between churning variables before and after hatching. There should also be a difference between developmental periods 2 and 3 because in developmental period 2 they can only partially escape the boundary layer, whereas in developmental period 3 individuals can lift off the gill arches for short periods of time. Developmental periods were then used to group days for the analysis of the external videotapes.

There was a developmental continuum in which a few of the young exhibited a novel behavior on a given day, but the majority (i.e. >50%) of young lagged behind. Developmental period 1 consisted of all days in which the majority of the young had not yet hatched. During developmental period 2 the majority of individuals had hatched but were not yet lifting themselves into the water column. Developmental period 3 consisted

of all days in which the majority were lifting themselves into the water column for short periods of time, and developmental period 4 consisted of all days that the majority of individuals were free-swimming.

Two mouthbrooding females (Individual 1 and Individual 2) were anesthetized using MS-222 (100-200 mg/L tricaine methanesulfonate). A hole was drilled into the preopercular bone through which a polyethylene cannula was inserted (2.15 mm i.d., 3.25 mm o.d., Intramedic PE 820) on Days 3 (Individual 1) or 6 (Individual 2). Activity inside the oral cavity was recorded for one brood on each of the females using a flexible fiberoptic endoscope (Olympus ultrathin fiberscope type 14, 1.4 mm o.d., 1.2 m working length, 75 degrees field of view, 0.2-5.0 cm depth of field) with a high intensity light source (Olympus Helioid ALS-6250) threaded through the cannula. A CCD video camera (Canon Ci-20R) connected with a Hi-8 video player/recorder (Sony EVO-9700, 30 frames s^{-1}) was attached to the endoscope.

Oral movement recordings and analysis

Oral movements were recorded to describe the pattern of these movements throughout the mouthbrooding period. The purpose was to (1) provide these results for another mouthbrooding species, (2) compare these variables with churning and ventilatory variables in other mouthbrooding species, (3) compare churning variables with fanning variables in substrate brooding species, and (4) provide a post-hoc explanation for the purpose of churning based on the changing patterns of these variables.

Using a hand-held Hi-8 camcorder (Sony CCD-TR81, 30 frames s^{-1}), oral movements were recorded daily for 15 consecutive min from Day 2 until the young were released at the end of the mouthbrooding period for each brood. One brood from each of three

individuals was tested in this manner (Individual 3; Individual 4, Brood 2; Individual 5). The duration of videotaping was increased to 20-30 min in two later broods on Individual 4 (Broods 2 and 3). All videotaping occurred from 1200-1600 hrs.

A 5-min segment of videotape (i.e. one sampling period) was selected for each day of each brood based on clarity of the videotape and a lower total number of breaks, defined as sections of videotape during which the fish was facing the back of the aquarium, as compared to other segments. Sections of videotape with less than 20 sec between breaks were excluded from analysis. The durations of breaks and the <20 sec-sections of videotape between breaks were summed. The segment of videotape analyzed for each day was then extended to give a full 5 min.

Each 5-min segment of videotape was viewed frame-by-frame using a Hi-8 video player/recorder (Sony EVO-9700) with a jog shuttle, and each oral movement was classified as either: pump, churn, or protraction (see Results section for description of oral movements). Protractions were excluded from analysis because fewer than 10 were observed throughout.

A bout was defined as an uninterrupted series of repititions of churns or of pumps. Analysis of each segment of videotape started at the beginning of the first churn or pump in the bout and ended after the last churn or pump of that bout. The start of a bout began on the first frame in which the mouth began to open, and the end of a bout was the frame before the mouth began to open for the other type of oral movement.

Type of oral movement, number of movements per bout, and time in frames per bout were recorded. From these values the following dependent variables for each type of oral movement or bout were calculated for each 5 min segment: the mean time per

movement, mean time per bout, mean number of movements per bout, and the mean number of each type of movement per 100 sec of videotape. Because only two oral movements were recorded, the mean time spent churning and pumping are inversely proportional. Therefore, only the mean time spent churning was recorded. For each churn bout there was a corresponding pump bout. Therefore, only the number of churn bouts per 100 sec was recorded. Positive and negative relationships between variables are illustrated in Figure 1.

Sampling periods that were characterized by long bouts necessarily resulted in a small total number of bouts. When another 5-min segment of videotape from the same day was analyzed, churning and pumping variables often differed markedly from the original section presumably because of a small, non-representative sample of long bouts. The combined total of all churning and pumping bouts was calculated for each brood, from which the mean for all sampling periods was determined. For sampling periods that had less than half of this mean number of bouts, additional videotape was analyzed to increase the total number of bouts for that period to one half of the mean. This procedure was used in the two broods for which 20-30 min were videotaped per day (Individual 4, Broods 2 and 3), resulting in an increase of a mean of 45.3 to a mean of 46.8 bouts per sampling period. Too little videotape was recorded in the first three broods (Individual 3; Individual 4, Brood 1; and Individual 5) to allow this procedure.

Bouts with 90 or more of either type of oral movement were excluded from analysis because these long bouts were not representative of a typical sampling period. In addition, these long bouts were often not quantifiable due to a large number of breaks. Presence or absence of these long bouts was recorded for each sampling period for each

brood. When another sampling period was analyzed from the same day, these long bouts were often not present. In two additional broods on Individual 4 (Broods 4 and 5), the presence or absence of these bouts was recorded by watching the individual for 60 min during each day of the mouthbrooding period. These bouts typically occurred as infrequently as three times per 60-min period.

Statistical analyses of oral movements

Repeated measures ANOVA was performed using JMP Version 2 (SAS Institute Inc., 1989) for all statistical analyses. A parametric two-way analysis for repeated measures allows individuals to be measured repeatedly over time and allows for one time variable to be nested within another (i.e. sampling periods within developmental periods) (Crowder and Hand, 1990). Mean values for each variable in each sampling period were grouped into one of the four developmental periods identified. The repeated measures were these values within each developmental period for each brood. The total number of days identified as belonging to a particular developmental period ranged from three to four. These unequal sample sizes among developmental periods are allowed by the repeated measures model (Crowder and Hand, 1990).

The repeated measures ANOVA was used to assess whether there were significant differences in oral movement and bout variables among the four developmental periods in one brood from each of three individuals (Individual 3; Individual 4, Brood 2; Individual 5). For Individual 4, one of the two broods for which 20-30 min were videotaped per day was selected randomly for this comparison among individuals. In addition, for this individual, repeated measures ANOVA was performed to assess whether there were significant differences in oral movement variables among the four

developmental periods in Broods 1, 2, and 3. The independent variable and main effect for all analyses was time. The random effect among individuals was individual, and within one individual the random effect was brood.

For these analyses, some untransformed and log-transformed or square root-transformed data were not significantly different from a normal distribution (Shapiro-Wilk test, $P>0.05$), and the variances were not significantly heterogeneous (Bartlett's test, $P>0.05$). For some variables, data were not significantly different from a normal distribution, and the variances were not significantly heterogenous only after excluding developmental period 3 or 4. Developmental periods 3 or 4 were chosen for exclusion because in order to evaluate the results in terms of proposed hypotheses for the function of churning, it was necessary to have results from developmental periods 1, 2, and either 3 or 4. Repeated measures ANOVA was then conducted using the data from the remaining developmental periods. If data were significantly different from a normal distribution and/or variances were significantly heterogenous after log-transforming, square root-transforming, and/or excluding developmental period 3 or 4, the repeated measures ANOVA was not conducted.

Two sequential Bonferroni tests (Rice, 1989) were used to adjust for an experiment-wise alpha value of 0.05. One was conducted for all churning and pumping variables combined among individuals (Individual 3; Individual 4, Brood 2; Individual 5), and another for these variables within one individual (Individual 4; Broods 1, 2, and 3).

The sample size was too small to provide accurate statistical outcomes. However, statistical tests were employed to provide a rough estimate of probabilities that differences occurred between developmental periods among individuals and within the

one individual tested with three broods. It is possible that the fish are not representative of this species, in which case I have misinterpreted the outcomes based on a biased sample.

There tended to be high levels of variability among individuals. Possible sources of variability included the age, size, brood experience, brood size, and the genetic relatedness of individuals. In addition, experimental procedures may have contributed to the variability between broods. The fish were obtained over approximately a two-year period of time. All fish were approximately the same age when acquired, therefore, two years was the maximum difference between individuals. Individuals ranged in size from 22-27 cm standard length. There was a wide range in brood experience among individuals, ranging from one to five successful brooding periods. Brood size ranged between individuals from approximately 100-1,000 young. The males were not related to any of the three females. However, two of the females may have been siblings. In the first three broods analyzed (Individual 3; Individual 4, Brood 1; Individual 5) temperature ranged from 26-28° C. Temperature was more closely controlled, ranging from 26-27° C, in Broods 2 and 3 from Individual 4. For these later broods, tank cleanings were more frequent and salt was added (0.06 g/L) to the aquaria.

Results

Endoscopic analysis

Eggs in the brooder's oral cavity formed a multi-layer heap. During the mouthbrooder's pumping bouts, the top layers of eggs were observed bouncing approximately 1/3 an egg diameter toward the roof of the oral cavity with each pump and then returned to their original position. After hatching, tails were observed beating rapidly but not continuously during pumping bouts. Independent movement of the young soon after hatching obscured the effects of pumping on individuals.

During churning bouts, the eggs and young were redistributed within the oral cavity. Due to rapid movement out of the field of view, the young could not be followed visually during churns long enough to determine if there was a direction to redistribution. From the first day some of the young had hatched until approximately Day 8, tails were observed beating rapidly and continuously during all churning bouts. Soon after Day 8, activity of the young made it difficult to see the effect of churning on tail beating.

On Day 4 of the mouthbrooding period in Individual 1, no young had hatched, and no independent movement of eggs was detected (data not available for Individual 2). In Individual 1, approximately 1/3 of the young had hatched on Day 5 as determined by the appearance of tails (data not available Individual 2). On Day 6 in both individuals, most of the young (i.e. >50%) had hatched. Immediately after hatching the tails were observed to beat rapidly at irregular intervals. Most hatched young were oriented with

their tails roughly perpendicular to the roof of the oral cavity, but a few had tails oriented parallel to the roof. Days 2-5 were designated developmental period 1 because Day 6 is the first day in which most young had hatched.

On Day 7 in both individuals, the young began to wriggle over each other. The first rapid oral movements of the young were detected on Days 8 (Individual 1) and 7 (Individual 2). On Day 9 in both individuals, most of the young had begun to lift off the gill arches for approximately 5-15 frames and hover momentarily before descending and contacting the gill arches or other young. Days 6-8 were designated developmental period 2 because Day 9 was the first day in which most of the young had begun to lift off the gill arches.

On Days 10 and 11, the young were able to hover for longer periods of time in the water above the gill arches. By Day 11 in Individual 1, a few individuals were free-swimming (data not available for Individual 2), differentiated from hovering by the absence of descending in the water followed by contacting the gill arches or other individuals. On Day 12, most of the young were free-swimming in both experiments. Some young were seen swimming outside of the brooder's oral cavity for the first time on Days 11 (Individual 1) and 12 (Individual 2). Days 9-11 were designated developmental period 3 because most of the young were free-swimming by Day 12.

Description of oral movements

No oral movements other than pumps, churns, and protractions were observed during the experiments. Pumps were defined as ventilatory movements in which water entered the oral cavity as the mandible was abducted (Fig. 2, A and B). The opercula remained adducted. The hyoid then abducted slightly as the mandible adducted (Fig. 2, C and D).

Water then exited via the abducted opercula (Fig. 2, E and F). Pumping in *O. esculentus* is similar to the description given by Oppenheimer and Barlow (1968) of active respiration in *S. melanotheron*.

Pump bouts with 90 or more pumps (Table 1) were present during all developmental periods (Individual 3) and developmental periods 1 and 2 (Individual 4). Individual 5 did not exhibit this behavior. Two churn bouts with 90 or more churns were detected in developmental period 2 for Individual 4, but were absent in all other individuals (Table 1).

Churns were divided into three phases. During Phase I of churns (Fig. 3, A and B) the mouth opened as the mandible was abducted, and the opercula remained adducted. Since this position is analogous to the mouth position during the beginning of a pump, water is assumed to be entering the mouth at this time. During Phase II (Fig. 3, C-F) the mandible was adducted and the opercula were abducted. Then the opercula were adducted as the mandible adducted completely, the premaxillae protruded, and the hyoid abducted. During Phase III (Fig. 3, G and H) the hyoid was adducted as the premaxillae retracted.

Consistent with the description of protractions in brooding *S. melanotheron* (Oppenheimer and Barlow, 1968), protractions differed from churns in that protraction of the premaxillae during closing of the mouth was accompanied by forward and lateral extension of the articulations between the premaxillae and the dentary, and this position was held for a prolonged period of time. A total of nine protractions was observed for Individuals 3 and 4. No protractions were observed for Individual 5.

Analyses among individuals

The mean time per churn bout and the mean time spent churning per 100 sec tended to increase from developmental period 1 to 2, then tended to decrease in developmental periods 3 and 4 (Figs. 3 and 4). The mean number of churns per bout exhibited a similar pattern to the mean time per churn bout, and the mean number of churns per 100 sec exhibited a similar pattern to the mean time spent churning per 100 sec. For these variables, there tended to be a high degree of variability in developmental periods 1 and 2 but little variability in developmental periods 3 and 4. Differences among developmental periods were not statistically significant, but the mean time per churn bout was significantly different among developmental periods ($P=0.02$, $F=7.7489$, df (3,6)) prior to the Bonferroni correction (Table 2).

The mean time per churn, mean time per pump, and the mean number of pumps per 100 sec tended to decrease from developmental period 1 to 2, then tended to increase in developmental periods 3 and 4 (Figs. 5, 6, and 7). For these variables, there tended to be a high level of variability in developmental period 4 but little variability in developmental periods 1 and 3. Only the mean time per churn could be analyzed using the repeated measures ANOVA because the other variables were significantly different from a normal distribution and/or significantly heterogenous even after log-transformation, square-root transformation, and/or exclusion of developmental period 3 or 4. For the mean time per churn, differences between developmental periods were not statistically significant (Table 2).

The mean time per pump bout tended to decrease from developmental period 1 to 4 (Fig. 8). The mean number of pumps per bout exhibited a similar pattern. For these

variables, there were no significant differences among developmental periods (Table 2). Developmental period 2 was highly variable.

The mean number of churn bouts per 100 sec tended to decrease throughout the brooding period (Fig. 9). Developmental periods 3 and 4 were highly variable. Differences among developmental periods were not statistically significant (Table 2), although this variable was significantly different among developmental periods ($P=0.045$, $F=5.1217$, df (3,6)) prior to the Bonferroni correction.

Analyses within one individual

Similar to the results among individuals, the mean time per churn bout and the mean time spent churning per 100 sec tended to increase from developmental period 1 to 2, then tended to decrease in developmental periods 3 and 4 (Figs. 10 and 11). The mean number of churns per bout exhibited a pattern similar to the mean time per churn bout, and the mean number of churns per 100 sec exhibited a pattern similar to the mean time spent churning per 100sec. For these variables, there tended to be a high level of variability in developmental period 2 but little variability in developmental period 4. The mean time spent churning per 100 sec could not be analyzed using the repeated measures ANOVA. For the remaining three variables, differences among developmental periods were not statistically significant (Table 3), although the mean number of churns per 100 sec was significantly different among developmental periods 1, 2, and 4 ($P=0.04$, $F=7.2359$, df (2,4)) prior to the Bonferroni correction. A peak in the mean time per churn bout occurred on Day 7 (Fig. 12). A peak in the mean time spent churning per 100 sec occurred on Day 8 (Fig. 13).

Similar to the results among individuals, the mean time per pump and the mean number of pumps per 100 sec tended to decrease from developmental period 1 to 2, then increase in developmental periods 3 and 4 (Figs. 14 and 15). For these variables, there tended to be a high level of variability in developmental period 2 but little variability in developmental period 4. Only the mean time per pump could be analyzed using the repeated measures ANOVA. For this variable, there was a significant difference ($P<0.001$, $F=22.6336$, df (3,6)) between developmental periods (Table 3). The mean time per pump tended to be lowest in developmental period 2 and highest in developmental period 4 (Fig. 14). The lowest and highest values were reached on Days 7 and 18 respectively (Fig. 16). The lowest value for the mean number of pumps per 100 sec occurred on Day 8 (Fig. 17).

Similar to the results among individuals, the mean time per pump bout tended to decrease from developmental period 1 to 4 (Fig. 18). The mean number of pumps per bout exhibited a similar pattern. For these variables, developmental period 2 was highly variable but developmental periods 3 and 4 exhibited little variability. Only the mean time per pump bout could be analyzed using the repeated measures ANOVA. For this variable, differences between developmental periods were not statistically significant (Table 3). A peak occurred in the mean time per pump bout on Day 4 (Fig. 19).

The mean time per churn and the mean number of churn bouts per 100 sec tended to remain low in developmental periods 1 and 2, then tended to increase in developmental periods 3 and 4 (Figs. 20 and 21). The mean number of churn bouts per 100 sec exhibited a pattern similar to the results among individuals except the mean time per churn which decreased from developmental period 1 to 2 among individuals (Fig. 5). For

these variables, there tended to be a low level of variability in developmental periods 1-3. Only the mean number of churn bouts per 100 sec could be analyzed using the repeated measures ANOVA. For this variable, a significant difference ($P<0.001$, $F=57.9665$, df (3,6)) was detected among developmental periods (Table 3). The lowest and highest values for the mean time per churn were reached on Days 2 and 18 respectively (Fig. 22). A peak occurred for the mean number of churn bouts per 100 sec on Day 16 (Fig. 23).

Day	Ind 3 (5 min)	Ind 4 (5 min)	Ind 4 (5 min)	Ind 4 (5 min)	Ind 4 (60 min)	Ind 4 (60 min)	Ind 5 (5 min)
2			P	P		P	
3					P	P	
4		P		P			
5	P	P		P	P	P	
6		P	C				
7			C				
8	P						
9	P						
10	P						
11							
12							
13	P						
14							
15							
16	P						
17							
18							
19							

Table 1. Presence of long ($\geq$ 90 movements) churn bouts (C) or pump bouts (P) in seven broods from three individuals.

Variable	Developmental Periods tested	P Value
mean time per churn	1, 2, 3, and 4	NS
mean time per churn bout	1, 2, 3, and 4	0.02
mean number of churns per bout	1, 2, and 3	NS
mean time spent churning	1, 2, 3, and 4	NS
mean number of churns	1, 2, and 4	NS
mean number of churn bouts	1, 2, 3, and 4	0.045
mean time per pump bout	1, 2, 3, and 4	NS
mean number of pumps per bout	1, 2, and 4	NS

Table 2. Churning and pumping variables for which repeated measures ANOVA could be conducted among three individuals. *= significant difference among developmental periods after the Bonferroni correction. NS: $P \geq 0.05$.

Variable	Developmental Periods tested	P Value
mean time per churn	1, 2, 3, and 4	NS
mean time per churn bout	1, 2, and 3	NS
mean number of churns per bout	1, 2, and 3	NS
mean number of churns	1, 2, and 3	0.04
mean number of churn bouts	1, 2, 3, and 4	<0.001*
mean time per pump	1, 2, 3, and 4	<0.001*

Table 3. Churning and pumping variables for which repeated measures ANOVA could be conducted among three broods from one individual. *= significant difference among developmental periods after the Bonferroni correction. NS: $P \geq 0.05$.

SPECIES	AUTHOR(S)	MEAN TIME/ CHURN BOUT OR FANNING BOUT	MEAN # CHURNS OR FIN BEATS/BOUT	MEAN TIME CHURNING OR FANNING
Mouthbrooding				
O. esculentus Cichlidae	current study	peak in developmental period 2	peak in developmental period 2	peak in developmental period 2
A. paraguayensis Cichlidae	Timms and Keenleyside, 1975			
S. melanotheron Cichlidae	Oppenheimer and Barlow, 1968	peak at end of developmental period 1	peak at end of developmental period 1	peak at end of developmental period 1
Substrate-brooding				
P. martensi Gobiidae	Torricelli et al., 1985	peak in developmental period 1	peak in developmental period 1	peak in developmental period 1
S. spinachia Gasterosteidae	Potts, 1984	peak in developmental period 1 or 2		
J. floridae Cyprinodontidae	Mertz and Barlow, 1966	peak at end of developmental period 1	peak at end of developmental period 1	peak in developmental period 1
B. badis Nandidae	Barlow, 1964	peak in developmental period 1 or 2	peak in developmental period 1 or 2	peak in developmental period 2
P. pungitius Gasterosteidae	Morris, 1958	peak in developmental period 1		peak in developmental period 1
C. gobio Cottidae	Morris, 1954			maximum values first reached in developmental period 1
B. soporator Gobiidae	Tavolga, 1954			downward
G. aculeatus Gasterosteidae	Van Iersel, 1953	peak in developmental period 1 or 2	peak in developmental period 1 or 2	peak at end of developmental period 1

Table 4. Trends exhibited in mouthbrooding and substrate brooding species for the mean time per churn bout or fanning bout, mean number of churns or fin beats per bout, and the mean time spent churning or fanning.

SPECIES	AUTHOR(S)	MEAN # CHURNS OR FIN BEATS/UNIT TIME	MEAN TIME/ CHURN OR FIN BEAT	MEAN # CHURN OR FANNING BOUTS/ UNIT TIME
Mouthbrooding				
O. esculentus Cichlidae	current study	peak in developmental period 2	lowest value reached in developmental period 2	increased in developmental periods 3 and 4
A. paraguayensis Cichlidae	Timms and Keenleyside, 1975		upward	
S. melanotheron Cichlidae	Oppenheimer and Barlow, 1968	peak at end of developmental period 1	increased after developmental period 1	began to increase at end of developmental period 2
Substrate-brooding				
P. martensi Gobiidae	Torricelli et al., 1985		upward	peak in developmental period 1
S. spinachia Gasterosteidae	Potts, 1984		lowest value reached in developmental period 1	peak in developmental period 1
J. floridae Cyprinodontidae	Mertz and Barlow, 1966	peak in developmental period 1	downward, plateaued in developmental period 1	peak in developmental period 1
B. badis Nandidae	Barlow, 1964	downward	peak in developmental period 1	downward, plateau reached in developmental period 1
P. pungitius Gasterosteidae	Morris, 1958			peak in developmental period 2
C. gobio Cottidae	Morris, 1954		peak at end of developmental period 1	
B. soporator Gobiidae	Tavolga, 1954		peak in developmental period 1 or 2	
G. aculeatus Gasterosteidae	Van Iersel, 1953		no change	peak in developmental period 1 or 2

Table 5. Trends exhibited in mouthbrooding and substrate brooding species for the mean number of churns or fin beats per unit time, mean time per churn or fin beat, and the mean number of churn or fanning bouts per unit time.

Fig. 1. Chronological sequence of video images illustrating a typical pump. Arrow in D indicates maximum hyoid abduction. The duration of the sequence is 0.7 sec.

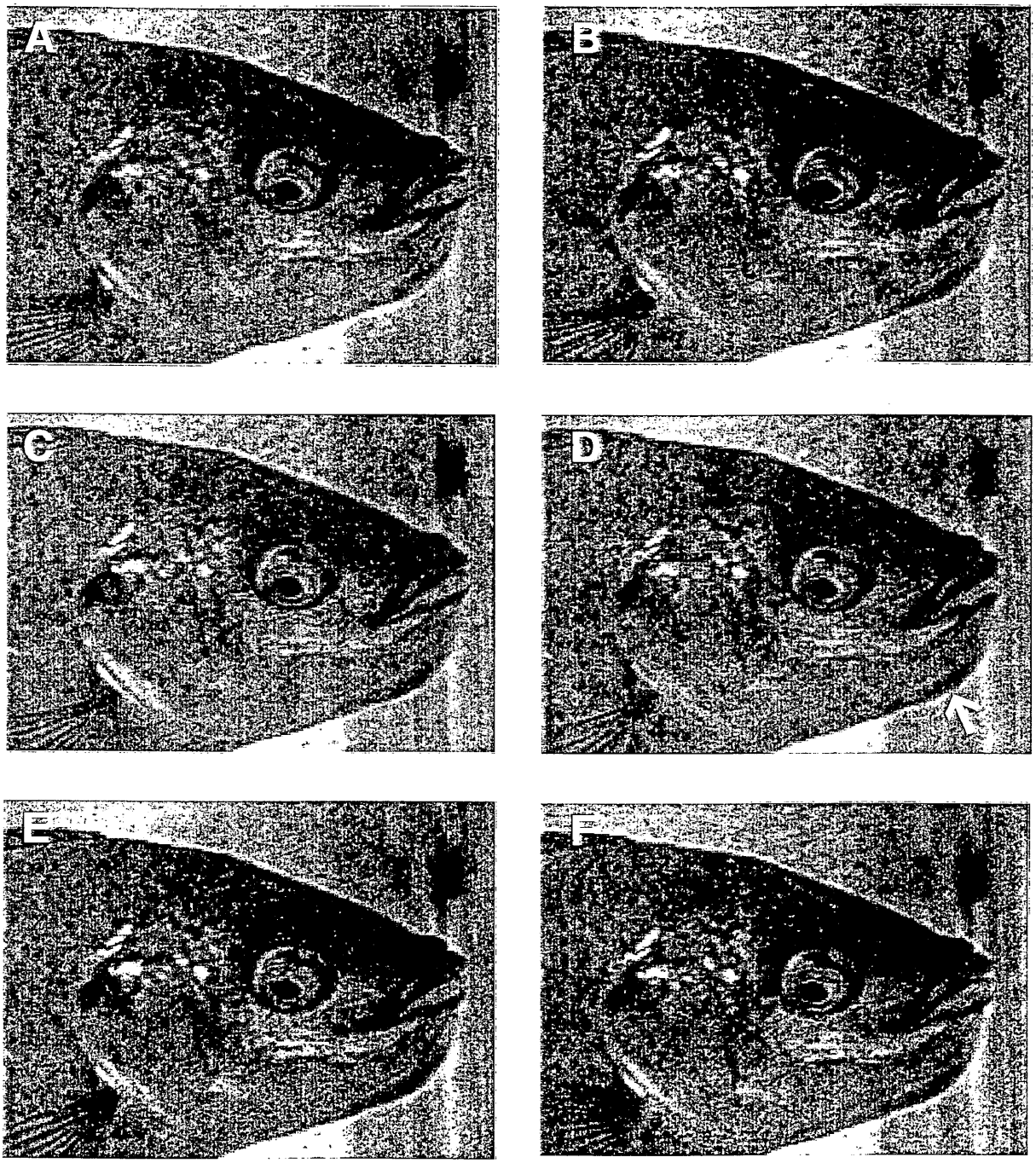
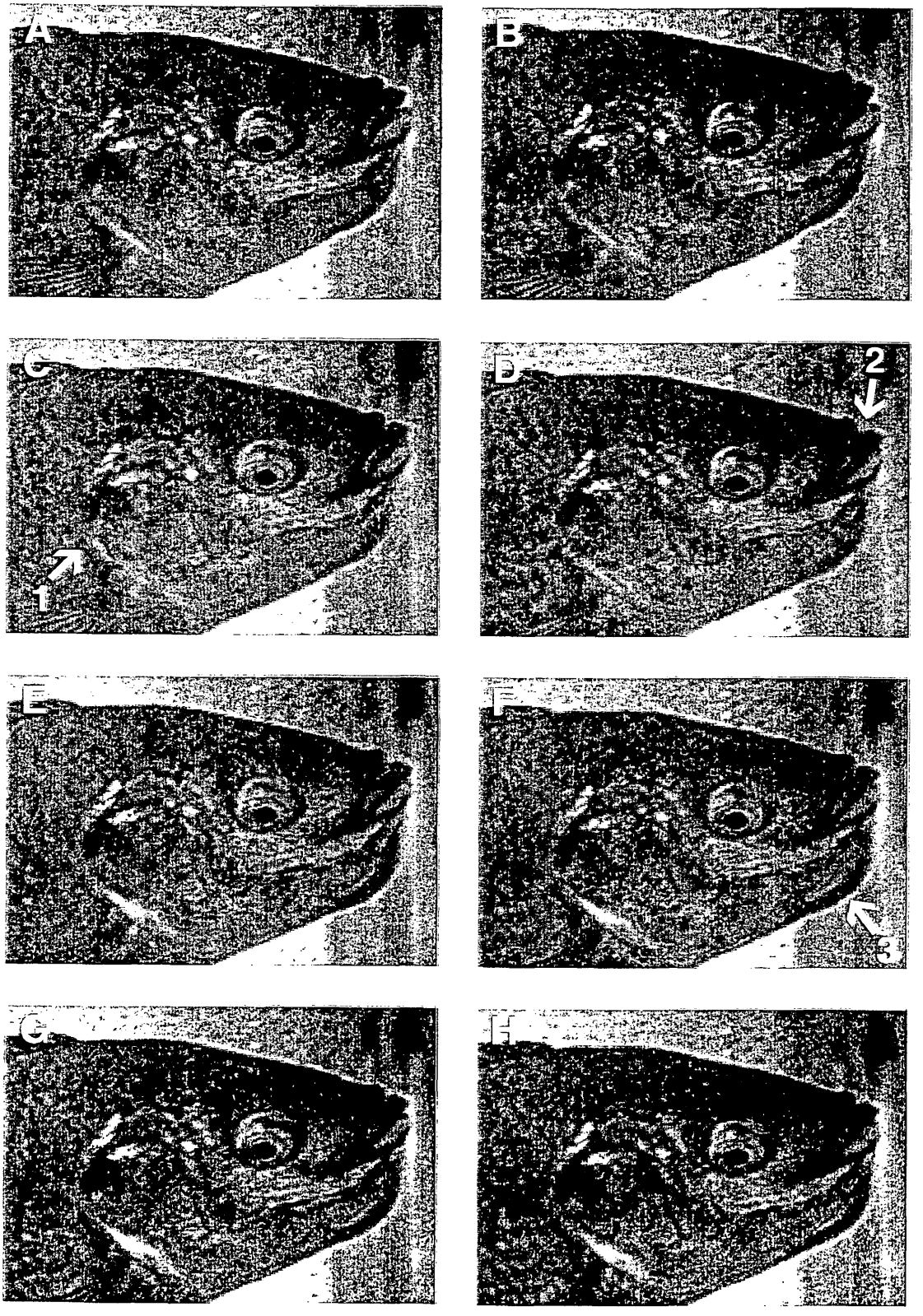

A
B
C
D
E
F

Fig. 2. Chronological sequence of video images illustrating a typical reversal. Arrow in C indicates maximum opercular abduction. Arrow in D indicates maximum premaxillary protrusion. Arrow in F indicates maximum hyoid abduction. The duration of the sequence is 0.5 sec.


A
B
C
1
D
2
E
F
3
G
H

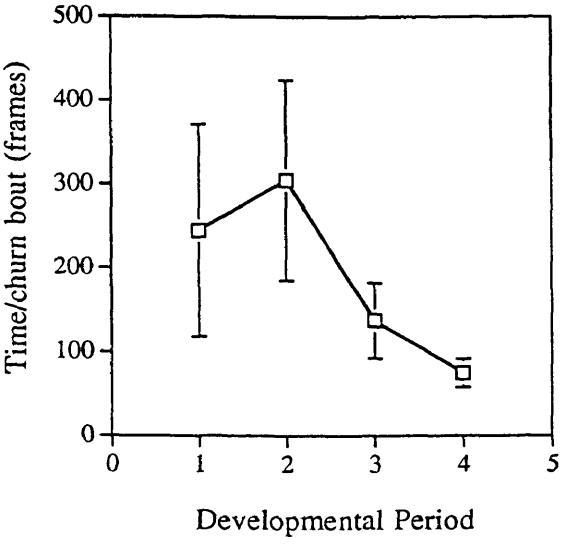


Fig. 3. Mean time per churn bout (± SE) for three individuals in each developmental period.

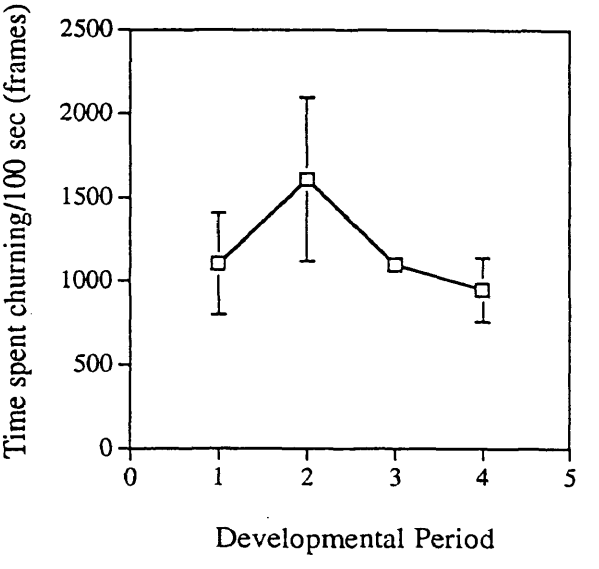


Fig. 4. Mean time spent churning per 100 sec of videotape (± SE) for three individuals in each developmental period.

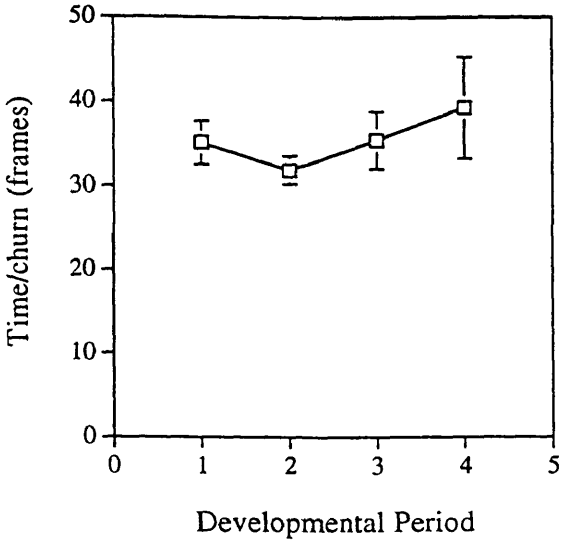


Fig. 5. Mean time per churn (± SE) for three individuals in each developmental period.

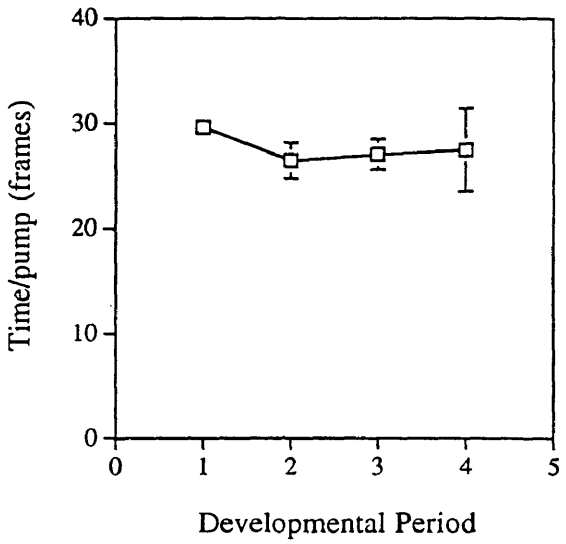


Fig. 6. Mean time per pump (± SE) for three individuals in each developmental period.

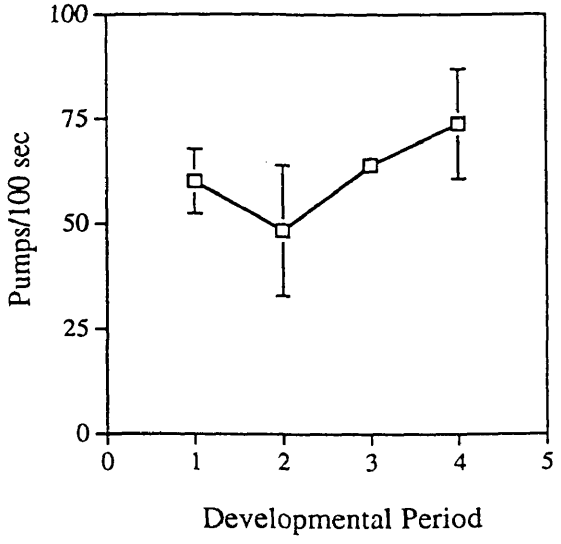


Fig. 7. Mean number of pumps per 100 sec of videotape (± SE) for three individuals in each developmental period.

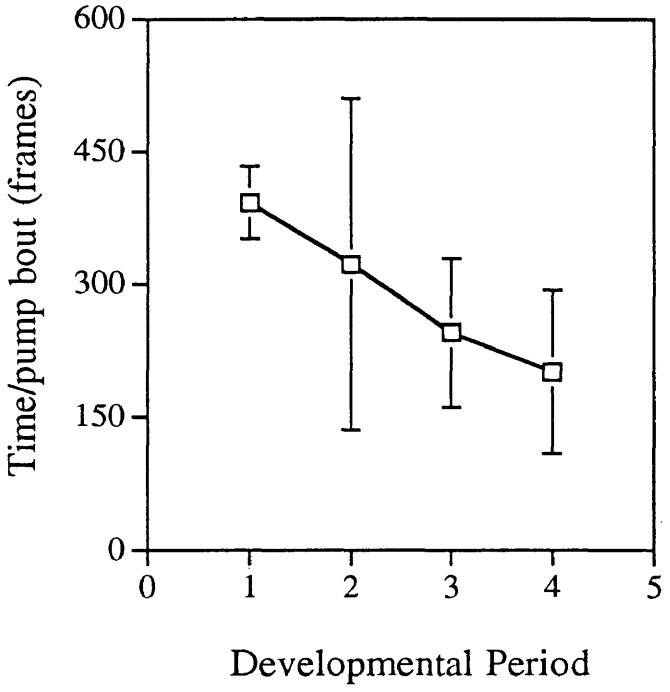


Fig. 8. Mean time per pump bout (±SE) for three individuals in each developmental period.

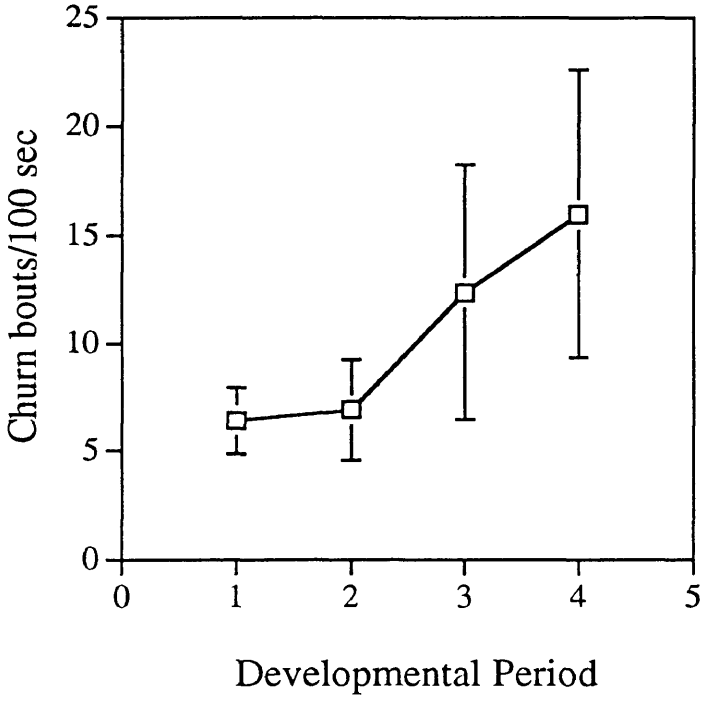


Fig. 9. Mean number of churn bouts per 100 sec of videotape (± SE) for three individuals in each developmental period.

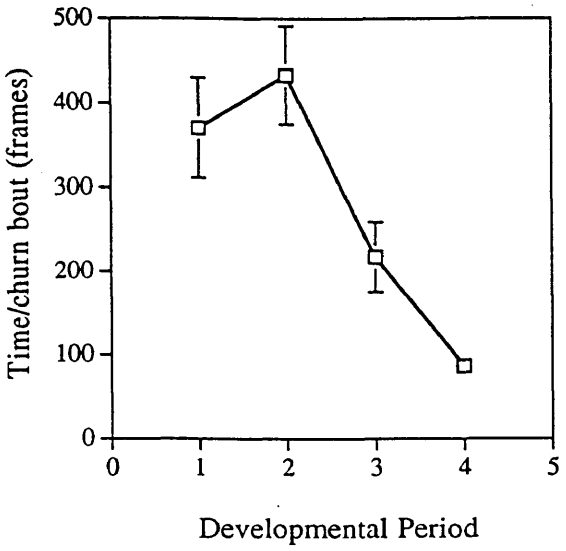


Fig. 10. Mean time per churn bout (± SE) for one individual in each developmental period.

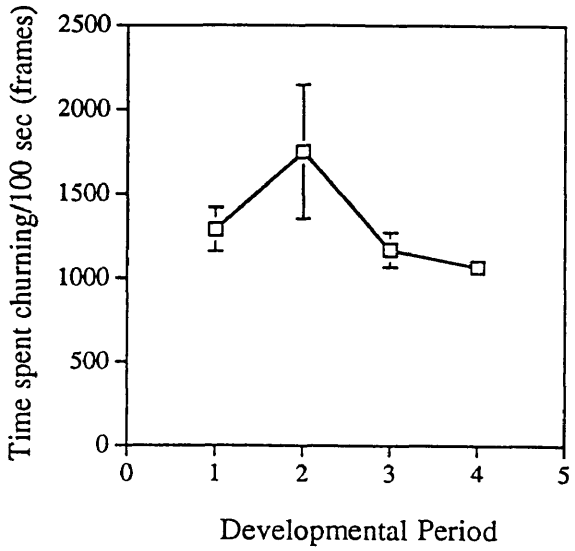


Fig. 11. Mean time spent churning per 100 sec of videotape (± SE) for one individual in each developmental period.

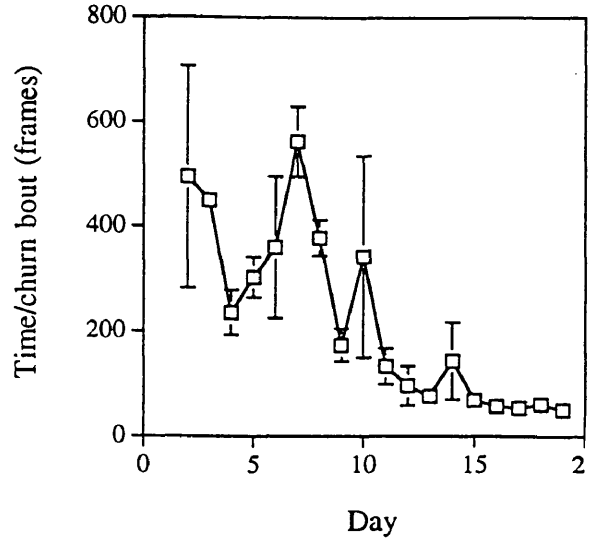


Fig. 12. Mean time per churn bout (± SE) for each day of the mouthbrooding period in one individual.

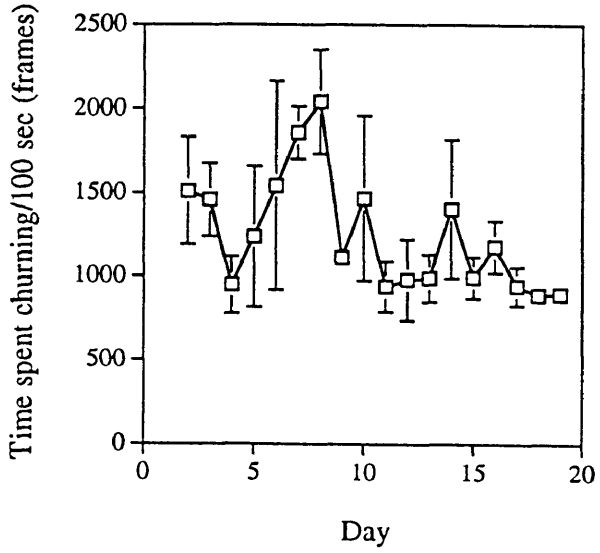


Fig. 13. Mean time spent churning per 100 sec of videotape (± SE) for each day of the mouthbrooding period in one individual.

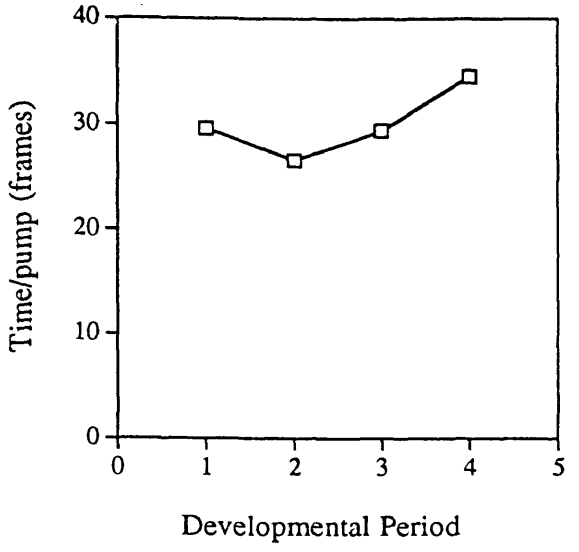


Fig. 14. Mean time per pump (SE too small to be shown) for one individual in each developmental period.

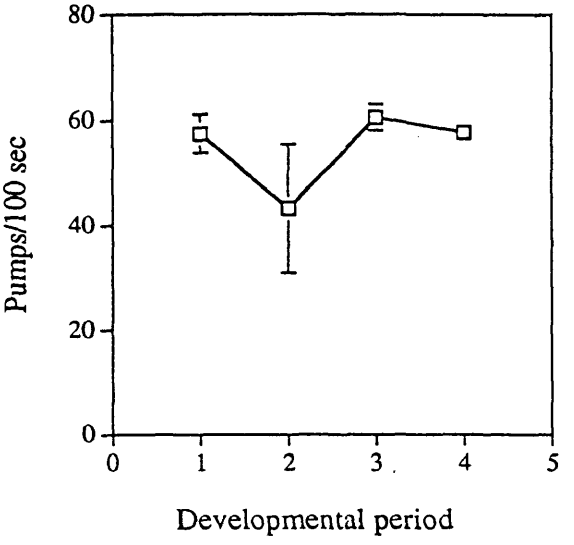


Fig. 15. Mean number of pumps per 100 sec of videotape (± SE) for one individual in each developmental period.

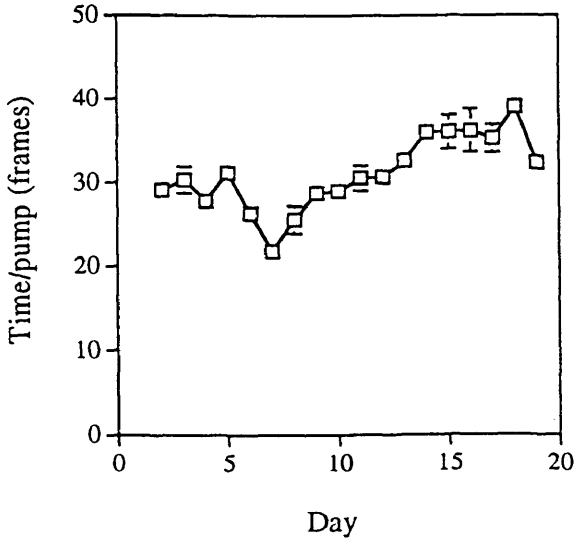


Fig. 16. Mean time per pump (± SE) for each day of the mouthbrooding period in one individual.

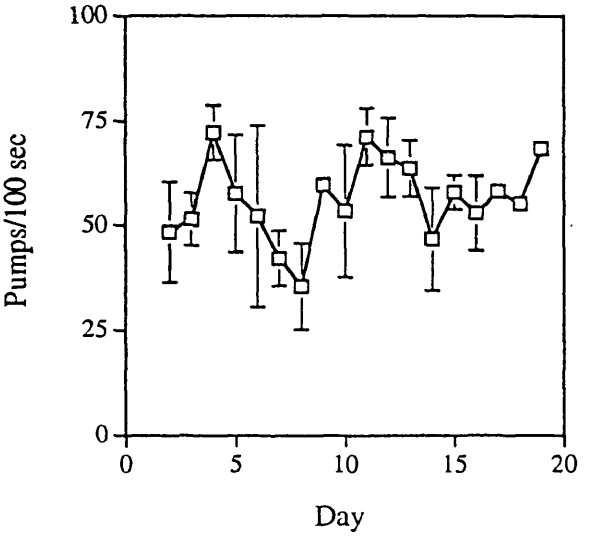


Fig. 17. Mean number of pumps per 100 sec of videotape (± SE) for each day of the mouthbrooding period in one individual.

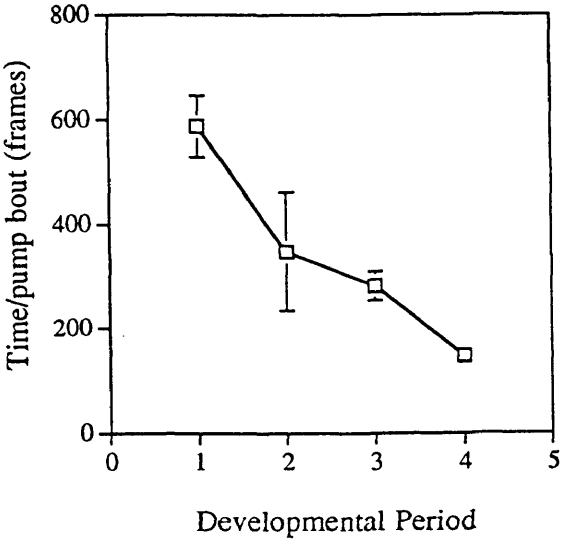


Fig. 18. Mean time per pump bout (± SE) for one individual in each developmental period.

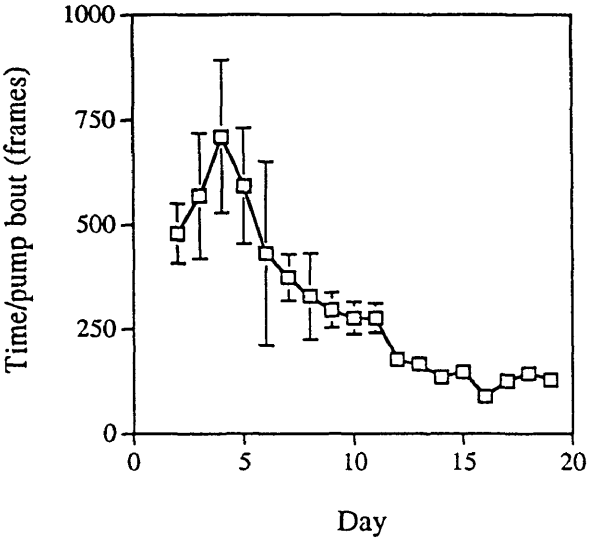


Fig. 19. Mean time per pump bout (± SE) for each day of the mouthbrooding period in one individual.

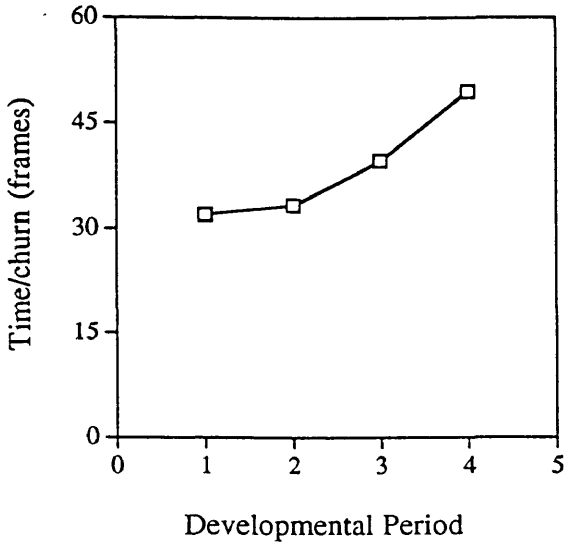


Fig. 20. Mean time per churn (SE too small to be shown) for one individual in each developmental period.

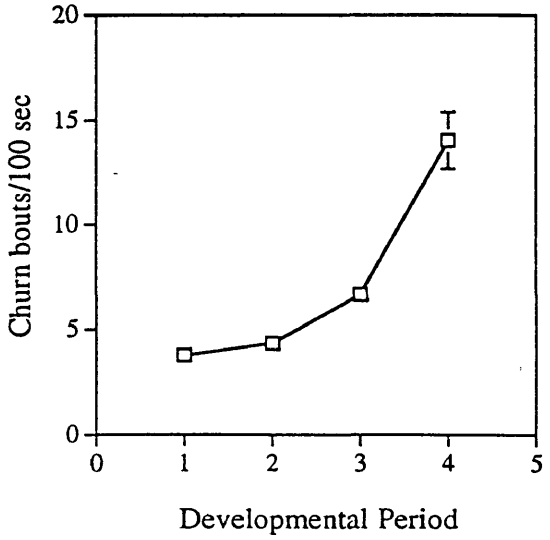


Fig. 21. Mean number of churn bouts per 100 sec of videotape (± SE) for one individual in each developmental period.

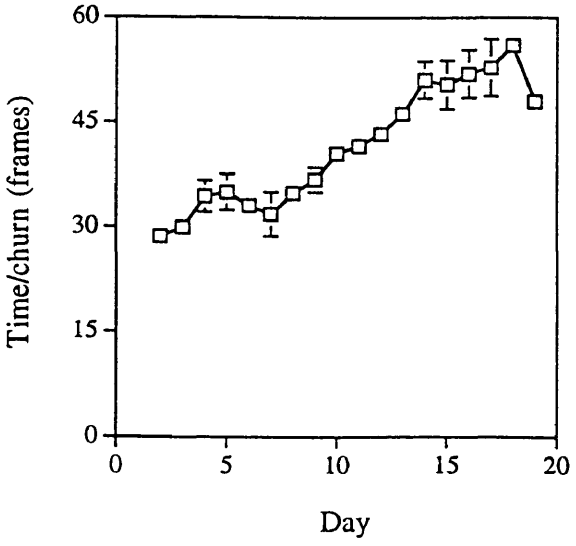


Fig. 22. Mean time per churn (± SE) for each day of the mouthbrooding period in one individual.

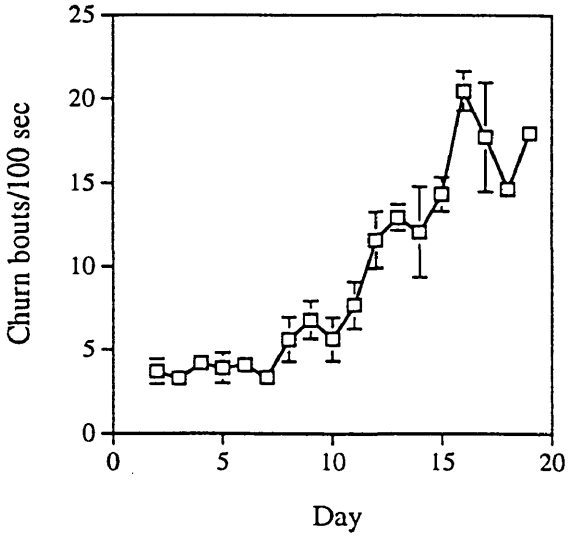


Fig. 23. Mean number of churn bouts per 100 sec of videotape (± SE) for each day of the mouthbrooding period in one individual.

Discussion

Endoscopic analysis

Only the top two layers of eggs were observed moving gently toward and away from the roof of the brooder's oral cavity during pumping bouts. Eggs underneath these top two layers could not be seen. However, because movement of the top two layers was slight, it is unlikely that eggs underneath these layers were able to penetrate the boundary layer and receive a fresh supply of water during pumping.

During churning movements the young beat their tails rapidly. This may be due to the presence of a respiratory plexus on the ventral finfold as was observed in post-hatching embryos of *Pseudocrenilabus philander* (Cichlidae) (Holden and Bruton, 1994), another mouthbrooding cichlid species. This activity may serve to increase the probability that an individual will move to or remain at the top of the heap where they are exposed to a current of fresh water, or it may facilitate oxygen exchange across the respiratory plexus during Phase II of the churning movement.

There is a developmental continuum in cichlid young that makes it difficult to determine the time when the young change from a non-free-swimming to a free-swimming condition (Keenleyside, 1991). Hatched, non-free-swimming young are referred to as "wrigglers." The term "fry" refers to free-swimming young (Keenleyside, 1991). These definitions are consistent with the developmental periods assigned in this study except that I divided the wriggler stage into two developmental periods,

developmental periods 2 and 3. In developmental period 2, individuals cannot lift off the gill arches. In developmental period 3, individuals are able to hover in the water column for short periods of time. I suggest that individuals in this third developmental stage be referred to as "bouncers" because they appear to lift off the gill arches for a few seconds, slowly descend, then bounce off the gill arches or their siblings.

Comparison with previous oral movement descriptions

The description of churns in *O. esculentus* is consistent with that of churns in *S. melanotheron* (Oppenheimer and Barlow, 1968), except hyoid movement was not mentioned. The description of churns in *O. esculentus* differs from that in *A. paraguayensis* (Timms and Keenleyside, 1975) in that complete mandibular adduction and adduction of the opercula were not mentioned (Timms and Keenleyside, 1975).

Rapid oral movements also occur when food is in the oral cavity (Oppenheimer and Barlow, 1968; Oppenheimer, 1970). These feeding movements have been termed reversals in *Oreochromis niloticus*, another immediate maternal mouthbrooding species (Onyari, 1983), because endoscopic analysis revealed water flow in the posterior to anterior direction during reversals (Sanderson et al., 1996). The description of reversals was similar to churning in *O. esculentus*. Abraham (1901) was able to observe externally the forward motion of individuals in the brooder's oral cavity during churning in *Pseudocrenilabrus philander* because that species has transparent regions on the throat and head. The results of these past studies suggest that water flow is probably in the posterior to anterior direction during brooding churns in *O. esculentus*.

Function of churning

The function of churning has most often been assumed to be aeration and waste removal (Hypothesis I) (Oppenheimer and Barlow, 1968; Oppenheimer, 1970; Timms and Keenleyside, 1975). There is direct evidence that fanning aerates the young because substrate brooders increase the mean time spent fanning, mean number of beats per bout, mean time per fanning bout, and the mean number of fanning bouts, and decrease the mean time per fin beat in response to environmental hypoxia during the egg stage (Jones and Reynolds 1999; Reebs et al., 1984; Takegaki and Nakazano, 1999; Torricelli et al., 1985) and throughout the brooding period (Potts, 1984). In addition, eggs of *Etroplus maculatus* (Cichlidae) (Zoran and Ward, 1983) exposed to faster rates of experimentally simulated fanning had a higher metabolism.

However, two other hypotheses have been proposed for which supporting evidence has been reported. The first is that churning prevents accumulation of heavy yolk lipids and subsequent deformation of embryos (Hypothesis II) (Timms and Keenleyside, 1975; Zoran and Ward, 1983). In the substrate brooding species *E. maculatus* (Zoran and Ward, 1983) the absence of simulated fanning resulted in greater accumulation of droplets presumed to be lipids underneath the yolk-sac of embryos compared to controls. Eggs that were not agitated often possessed deformities and failed to hatch (Zoran and Ward, 1983). Another hypothesis is that churning or fanning prevents fungal infection by keeping the eggs clean (Hypothesis III) (Côté and Gross, 1993; Timms and Keenleyside, 1975). After hatching this function is thought to become less important because the locomotor abilities of the young increase (Timms and Keenleyside, 1975). In the substrate brooding species *Lepomis macrochirus* (Centrarchidae), colonial males

devoted more time to fanning than solitary males, and fungal infection by *Saprolegnia* species was less prevalent among the eggs of colonial males (Côté and Gross, 1993). Although neither a substrate brooding nor mouthbrooding species, the eggs of *Oncorhynchus mykiss* (Salmonidae) (Rach et al., 1995) were less likely to become infected with fungus when they were experimentally rolled at higher rates than controls.

Endoscopic analysis revealed that churns are responsible for redistribution of the young in the oral cavity of *O. esculentus*. An observed result of churns is, therefore, mixing of the young. Because of the large number of individuals in the oral cavity, each churn is likely to cause only partial mixing of the young. There are a number of strategies a mouthbrooder may utilize to redistribute the young more thoroughly. All churning variables except for the mean time per churn and the mean number of churn bouts per 100 sec may increase when redistribution is most critical. The mean time per churn may decrease because a faster movement is likely to increase the flow rate of water in the brooder's oral cavity, and the mean time per pump bout may decrease because this results in a shorter period of time between churning bouts. Because the mean time per churn bout may increase while the mean time per pump bout may decrease, the mean number of churn bouts per 100 sec may remain relatively unchanged. However, in substrate brooders a peak may occur in the mean number of fanning bouts at hatching because this behavior does not interfere with ventilation of the adult.

Churning and pumping variables are related by a variety of positive and negative relationships. Because a particular variable may be positively related to one variable and negatively related to another, a number of different trends may be exhibited by these variables. For example, if the mean number of churns per bout increases and the mean

time per churn bout remains constant, then the mean time per churn will decrease. However, if the mean number of churns per bout increases and the mean time per churn remains constant, then the mean time per churn bout will increase. In addition, effectiveness of redistribution is often linked among variables. For example, increasing the mean number of churns per bout will not necessarily facilitate redistribution if each churn is longer in duration. Because there are a large number of strategies a mouthbrooder may employ to maximize redistribution, it is possible that only certain strategies are utilized and that patterns in other variables are the result of relationships with the variables involved in these strategies.

The oxygen requirements of the young increase throughout the brooding period (Lindström and Wennström, 1994;Oppenheimer and Barlow, 1968; Oppenheimer, 1970; Takegaki and Nakazano, 1999). However, the young cannot escape the boundary layer to receive fresh water entering the parent's mouth until developmental period 3 when they begin to lift off the gill arches. Similarly, the amount of waste produced by the young increases throughout the brooding period, but before developmental period 3 the young are trapped beneath the boundary layer where they are exposed to wastes.

If aeration and waste removal are the primary purposes of churning (Hypothesis I) then a peak should occur during developmental period 2 in churning variables except the mean time per churn, and the lowest values for the mean time per churn and the mean time per pump bout should occur in developmental period 2. However, if prevention of the accumulation of heavy yolk lipids (Hypothesis II) or prevention of fungal infection (Hypothesis III) is more important, then there should be a downward trend among churning variables except the mean time per churn, while the mean time per churn and

the mean time per pump bout should increase throughout the brooding cycle as the locomotor abilities of the young increase (Timms and Keenleyside, 1975).

Comparison with previous oral movement analyses

Churning variables and their fanning correlates were compared among all ten reports of substrate brooding and mouthbrooding species for which some or all of these variables were measured daily throughout the brooding period. The mean time per pump bout was compared with the mean time per active respiration bout in *S. melanotheron*. The purpose of these comparisons was to evaluate churning, fanning, and ventilatory variables in terms of the hypotheses aforementioned.

All but one past study (Keenleyside and Bietz, 1981) did not use descriptions of development and behavior of the young to assign developmental periods. The approximate time of hatching was reported in all past studies, but hatching was loosely defined making it difficult to determine whether the day of hatching corresponded to the first day on which individuals started to hatch, or the day by which the majority or all individuals had hatched. Consequently a peak in a particular variable "at the time of hatching" in previous studies may have corresponded to developmental periods 1 or 2 of this study because developmental period 2 began on the day that the majority of individuals had hatched. Some authors described hatching as the "first estimated day of hatching" which corresponded to the end of developmental period 1 in this study.

Keenleyside and Bietz (1981) examined fanning behavior across three developmental periods, egg, post-hatching embryos, and free-swimming young in the substrate brooding species *Aequidens vittatus* (Cichlidae). Values for the mean time spent fanning, mean fanning bout frequency, and the mean time per fanning bout were highest in the egg

stage. However, these variables were not measured for each day of the brooding period. Therefore, it is not known whether a peak in these variables occurred in developmental period 1 or 2 or whether there was a downward trend in these variables throughout the brooding period. These results were not included in the following analysis because they are not directly comparable to this study or past studies.

All study species for which the mean time per churn bout or fanning bout, and the mean number of churns or fin beats per bout were reported exhibited a peak in these variables during developmental period 1 or 2 (Table 4). Eight of the nine study species for which the time spent churning or fanning was reported including *O. esculentus* exhibited a peak or first reached maximum values for these variables during developmental period 1 or 2 (Table 4). Three of four study species including *O. esculentus* exhibited a peak in the mean number of churns or fin beats per unit time in either developmental period 1 or 2 (Table 5). These results are consistent with Hypothesis I.

Mouthbrooding and substrate brooding species for which the mean time per churn or fin beat was reported exhibited a variety of patterns (Table 5). Two of ten study species including *O. esculentus* reached minimum values for the mean time per churn or fin beat in either developmental period 1 or 2. These results support Hypothesis I. However, two species exhibited an upward trend in these variables, which supports Hypotheses II and III. Six study species exhibited patterns in the mean time per churn or fin beat which did not support Hypotheses I, II, or III (Table 5).

In *O. esculentus* and *S. melanotheron* the mean number of churn bouts per 100 sec remained relatively constant in developmental periods 1 and 2, then increased in developmental periods 3 and 4 (Table 5). This pattern does not follow my prediction that

this variable would remain relatively unchanged in mouthbrooding species. In support of Hypothesis I, five of six substrate brooding species for which this variable was reported exhibited a peak in the mean number of fanning bouts in either developmental period 1 or 2.

In *O. esculentus* the mean time per pump bout exhibited a downward trend throughout the mouthbrooding period. In *S. melanotheron*, the mean time per active respiration bout decreased in developmental period 1, then remained relatively constant in later developmental periods (Oppenheimer and Barlow, 1968). These findings do not support any of the proposed hypotheses. However, a decrease in this variable and an increase in the mean time per churn bout from developmental period 1 to 2 supports Hypothesis I. The increase in the mean number of churn bouts in developmental periods 3 and 4 was the result of having short pump and churn bouts. A combination of short pump and churn bouts may be a strategy utilized by the mouthbrooder to keep the young in the mouth while they are swimming against a current. In addition, oxygen levels are likely to be higher toward the front of the oral cavity. Frequent churn bouts may redistribute the young so that those in the back of the oral cavity are brought to the front where they are exposed to higher levels of oxygen.

The only variables that were significantly different between developmental periods were the mean number of churn bouts per 100 sec and the mean time per pump for the one individual in which 3 broods were tested. However, there were trends (i.e. significant differences between developmental periods before the Bonferroni correction) in the mean time per churn bout and the mean number of churn bouts per 100 sec among individuals, and the mean number of churns for the one individual tested with 3 broods.

Perhaps the best strategy to redistribute the young during developmental periods 1 and 2 is to increase the mean time per churn bout, resulting in few long bouts. In later developmental periods, there may be many short bouts because frequent and not prolonged redistribution of the young is favored. A shorter mean time per pump probably results in a faster ventilatory rate which may be required to adequately oxygenate the parent when they are spending the most time churning in developmental periods 1 and 2. Adult fish can increase their ventilatory rate in response to environmental hypoxia (Greco et. al., 1995; Massabuau and Forgue, 1995). In addition, a shorter mean time per pump may provide a faster flow rate of water to the young when oxygenation of the young is most critical. The other churning variables exhibited a pattern similar to the mean time per churn, reaching a minimum in developmental period 2. The other pumping variables exhibited patterns similar to the mean time per pump or the mean time per pump bout, decreasing throughout the mouthbrooding period. It is not known whether the patterns of these other churning and pumping variables held biological significance or whether they were the result of correlations with the variables that were significantly different between developmental periods before or after the Bonferroni correction.

Thus, the results for most churning variables in *O. esculentus* were similar to the patterns observed in churning in other mouthbrooding species and fanning in substrate brooding species. A peak or minimum value reached in predicted churning and pumping variables during developmental period 2 in *O. esculentus* is consistent with the hypothesis that aeration and waste removal are the primary purposes of churning. Similarity of churning variables in *O. esculentus* to fanning bout variables in substrate

brooding species, for which there is direct evidence that fanning ventilates the young, is also consistent with this hypothesis.

These conclusions should be considered with caution because of the small sample size. The results for one individual in which three broods were tested exhibited strong trends and were relatively consistent between broods; however, it is not known if this individual is representative of its species. There was tremendous variability between individuals. It is not known whether this variability is because one or more of the individuals is not representative of the species, because experimental conditions were not closely controlled, or whether this variability is a reflection of the variability present in the species. The weak trends observed among individuals, which were also consistent with stronger trends exhibited within the one individual, were interpreted as being biologically significant.

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